1636

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application Number	09/297,092
Filing Date	May 18, 1999
Inventor	PAULISTA et al.
Group Art Unit	1636
Examiner Name	S. Kaushal
Attorney Docket Number	2923-0115

Title of the Invention: COMPOUNDS WITH IMPROVED CARTILAGE-INDUCING AND/OR BONE INDUCING ACTIVITY

Transmittal of Declaration Under 37 C.F.R. 1.132

Commissioner for Patents
Washington, D.C. 20231

April 22, 2003

Dear Sir:

Further to the response filed on March 24, 2003 and the supplemental response filed on March 27, 2003, attached is the signed declaration referenced in the responses.

An unsigned copy was previously filed for the Examiner's review.

RESPECTFULLY SUBMITTED,					
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SIGNATURE	M. C. Kitts		*DATE*	4/22/03	
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Enclosure: Declaration

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DECLARATION UNDER 37 C.F.R. 1.132

Commissioner for Patents
Washington, D.C. 20231

Sir:

I,Gertrud Hötten, hereby state and declare as follows:

I am very familiar with the present invention, the above-identified application, and the Office Action dated October 23, 2002.

Experiments were conducted under my direction and control which show that MP52 has cartilage and bone inducing activity. The experiments which were conducted are as follows.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed this 4 day of April 2003

~~Dr.~~ Gertrud Hötten

Expression of MP52 in E.coli

A part of MP52 containing additional Histidine residues at its N-terminus (HisMP52) was expressed in E.coli. The His-tag simplifies the purification by binding to metal chelat columns.

A C-terminal part of MP52 (119 amino acids) containing the amino acids 383-501 in SEQ ID NO. 1 and additional 10 amino acids at the N-terminus (MHHHHHHKLI) was expressed using the prokaryotic vector pBP2. The vector pBP2 is a derivative of the pBR322 plasmid containing an ampicillin resistance gene. The T7-promoter is followed by a ribosome binding site, a start codon, 6 Histidine codons, a multiple cloning site for insertion of the target gene, stop codons in each reading frame and a terminator. The plasmid containing the above mentioned part of MP52 was deposited at the DSM (DSM 10028, 2. Juni 1995). The expression of HisMP52 is induced by providing a source of T7 RNA polymerase. The expression host BL21(DE3)pLysS (Novagen #69451-1) contains a chromosomal copy of the T7 RNA polymerase gene under lacUV5 control and expression of HisMP52 was induced by IPTG according to the instructions of the manufacturer. Monomeric HisMP52 is expressed in inclusion bodies which can be isolated according to standard procedures. Purification of HisMP52 was performed using a Nickel chelat column as described by Hochuli et al. (BIO/Technology 6, 1988, 1321-1325). Further purification was done by a reversed phase column (Nucleosil 300-7C4, Machery-Nagel, 715023) with a 0 to 90% acetonitril gradient containing 0.1% TFA in 100 minutes (flow rate: 2 ml/min). The elution of HisMP52 starts at about 35% acetonitril. The lyophilized HisMP52 was solubilized in a denaturing buffer (6M guanidinium chlorid, 150 mM NaCl, 3 mM DTT, 10 mM Tris pH 8; 2,6 mg/ml) and refolded to the dimeric HisMP52 at a final concentration of 160 µg/ml in a common Tris-buffer system (pH 9.5) containing EDTA (2-10 mM), CHAPS (15-50 mM), NaCl (1-2 M) and a redox-system (1 mM GSSG, 2 mM GSH) for 48 hours at 23°C. Residual monomeric HisMP52 was separated from the dimer by reversed phase HPLC. For this purpose HisMP52 was loaded on the column (Aquapore Octyl 20 micron, Applied Biosystems) at 35% buffer B (buffer A: 0.1% TFA in water, buffer B: 90% acetonitril, 0.1 % TFA). With a 35-60% buffer B gradient in 50 minutes (flow rate 3 ml/min) the

dimeric HisMP52 starts to elute at about 40% buffer B followed by the monomeric form starting at about 43% buffer B. The purified dimeric HisMP52 was lyophilized, stored at -70°C and used for the biological activity studies.

Additionally a C-terminal part of MP52 (119 amino acids) containing the amino acids 383-501 in SEQ ID NO. 1 were expressed and purified essentially as described in detail in the WO 96/33215. This protein, starting with a Proline at its N-terminus was named rhMP52 (recombinant human MP52).

Biological activity of MP52

***In vivo* parietal bone assay**

HisMP52 (1, 3 and 10 µg/20µl/site) was dissolved in phosphate-buffered saline (PBS; pH 3.4) containing 0.01 % human serum albumin and repeatedly injected onto the periosteum of neonatal rat parietal bone once a day. The injection of HisMP52 was started one day after birth and finished after 12 days of injection for histopathological examination (hematoxyline-eosin stain).
As shown in FIGURE 1, HisMP52 stimulates in a dose dependent manner the increase of bone thickness by newly formed bone.

***In vivo* segmental bone defect model**

A five millimeter segmental bone defect was created in a middle region of the femur of 13-week-old male Sprague-Dawley rats using a fine toothed saw blade. Physiological saline was dropped for avoiding tissue damage. A polyethylene plate was fixed along the lateral cortex with 2 millimeter diameter stainless screws. A solution containing 0.5% porcine type I collagen (200 µl) was mixed with HisMP52 (20µg), lyophilized and implanted into the defects. A solution of 0.5% porcine type I collagen alone was treated in the same manner and used as a control. The rats were sacrificed 12 weeks after the implantation and the femora were removed.

The time course of healing of the segmental bone defects was evaluated by soft X-ray radiography after 4, 8 and 12 weeks of implantation. As shown in FIGURE 2 the defect treated with HisMP52 was filled with mineralized tissue after 8 and 12 weeks whereas the control caused no radiographical changes in the defect even after 12 weeks.

The bone mineral content in the defects of the femurs was measured after the 12-week treatment by dual energy X-ray absorptiometry (DEXA). The HisMP52 group has a significantly elevated level compared to the control group as shown in FIGURE 3.

One rat of both groups was subjected to histological analysis after 12 weeks. Staining (hematoxyline and eosin, alcian blue) of decalcified sections of the femur defects treated with HisMP52 revealed an accomplished osseous union across the defect containing bone marrow cells (data not shown). In the control, muscle, adipose and fibrous tissues showed only a delayed or non-union defect.

For measuring the torsional strength the polyethylene plates and stainless screws were removed and the diaphyses of the femurs of the remaining rats were fixed by burying up both ends with resin and attaching them to the bone strain system (MZ-500D, Maruto Testing Machine Co.). The lower resin was rotated at a speed of 180 degrees/min. The torsional strength was determined by measuring the maximum force required to break the bone. As shown in FIGURE 4, the torsional strength of the femurs treated with HisMP52 is significantly higher then the control value.

Full-thickness defect model of rabbit articular cartilage

A two millimeter-diameter defect was created in the medial femoral condyle through the subchondral plate in rabbits (about 2 kg) with an orthopedic hand drill. After that, the drill hole was reamed with a biopsy needle. The hyaluronic acid gel (10µl, 1%) mixed with or without HisMP52 (3 µg) was introduced into the cartilage

defect. Six weeks after operation, decalcified transections of the articular cartilage were stained with alcian blue.

FIGURE 5 shows that a zonal structure appears after treatment with the HisMP52/ hyaluronic acid (HA) mixture which resembles the intact cartilage. The HA-treated control defects show the generation of chondrocytes but miss the zonal structure of articular cartilage.

Ectopic bone formation assay in mice

The ectopic bone formation assay is a well known method to determine the cartilage and bone inducing potential of BMPs or related proteins. Cerasorb® is a crystallographically pure ß-TCP matrix and was used with a granule size of 50-150 µm and 150-500 µm. Cerasorb® was coated with 200 µg rhMP52 and was implanted subcutaneously in the mice (male ICR, 8 weeks) backs. 200 µg rhMP52/ß-TCP (50-150 µm) were implanted in 5 mice and 200 µg rhMP52/ß-TCP (150-500 µm) were implanted likewise in 5 mice. The administration sites were dissected two weeks after implantation and submitted to histological examination. FIGURE 6 shows that both rhMP52/ß-TCP combinations with different granule sizes were able to induce new bone.

The above described experiments clearly demonstrate, that MP52 is very useful for treating bone damage or defects as well as diseases which can be diminished or healed by bone and cartilage growth. These could be for example treating of bone fractures and non unions, bone reconstruction, applications in the jaw, dental or facial region and spinal fusions.

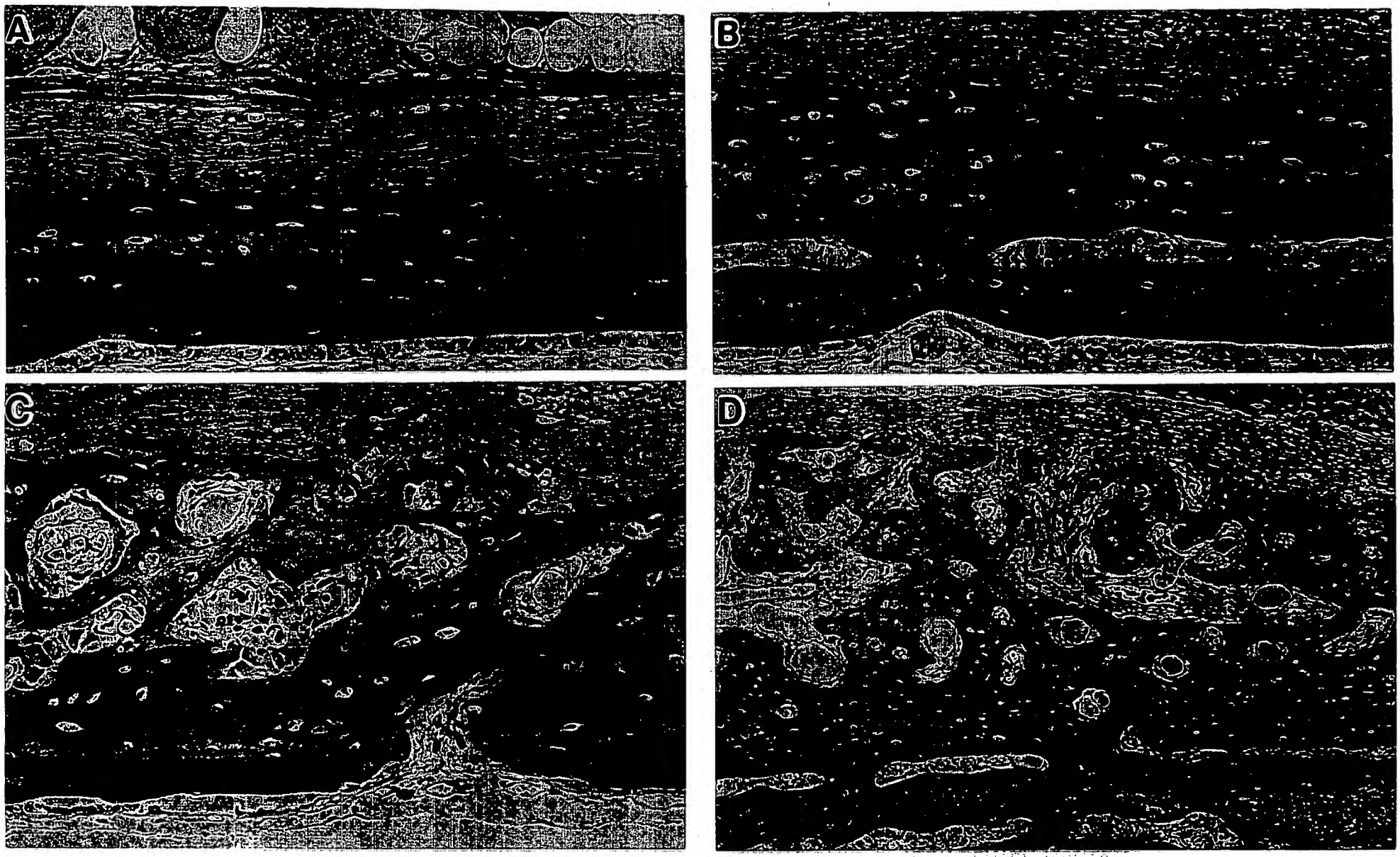

FIGURE 1: Calvaria from neonatal rats treated with HisMP52 (B: 1 µg; C: 3 µg; D: 10 µg). The control is shown in A.

Magnification x 66 (A, B, C), x33 (D).

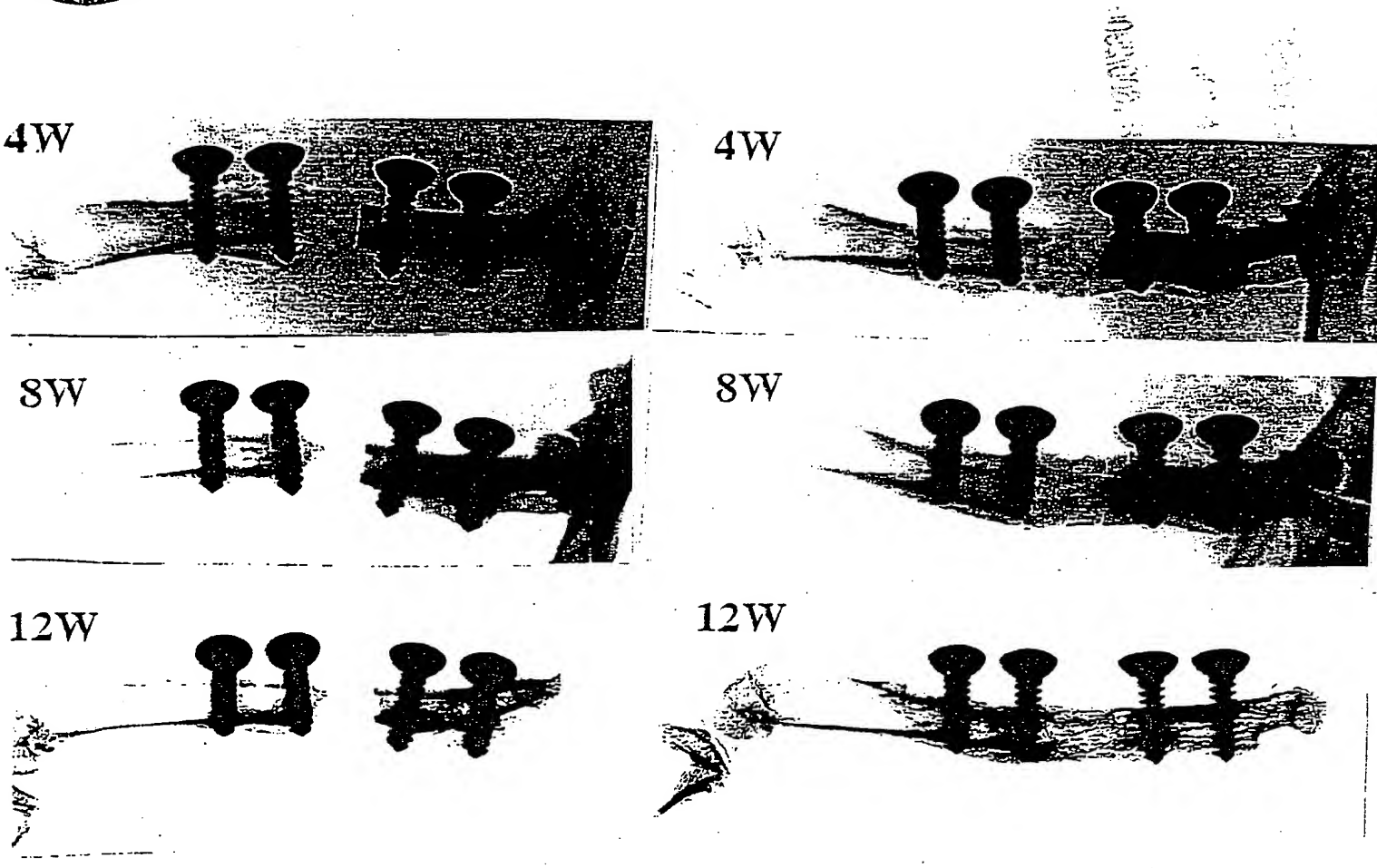

FIGURE 2: Radiographic changes in segmental bone defects (5 mm) in rat femurs 4, 8 and 12 weeks (w) after implantation of HisMP52 (20 μg/site) with type I collagen fibers or collagen fibers alone as a control.

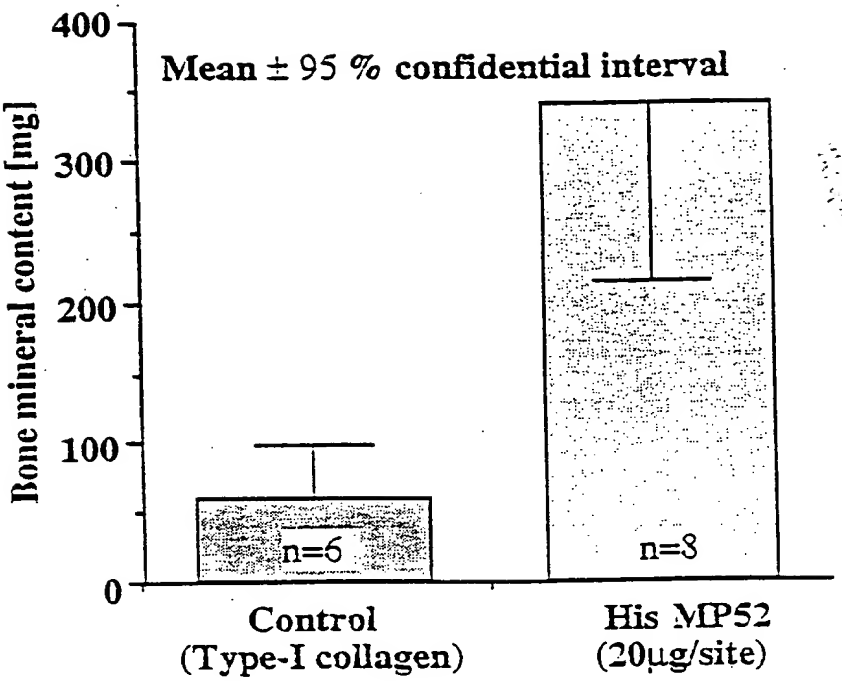

FIGURE 3: Bone mineral content of segmental bone defects (5 mm) in rat femurs 12 weeks after implantation of HisMP52 (20 µg/site) with type I collagen fibers or collagen fibers alone as a control.

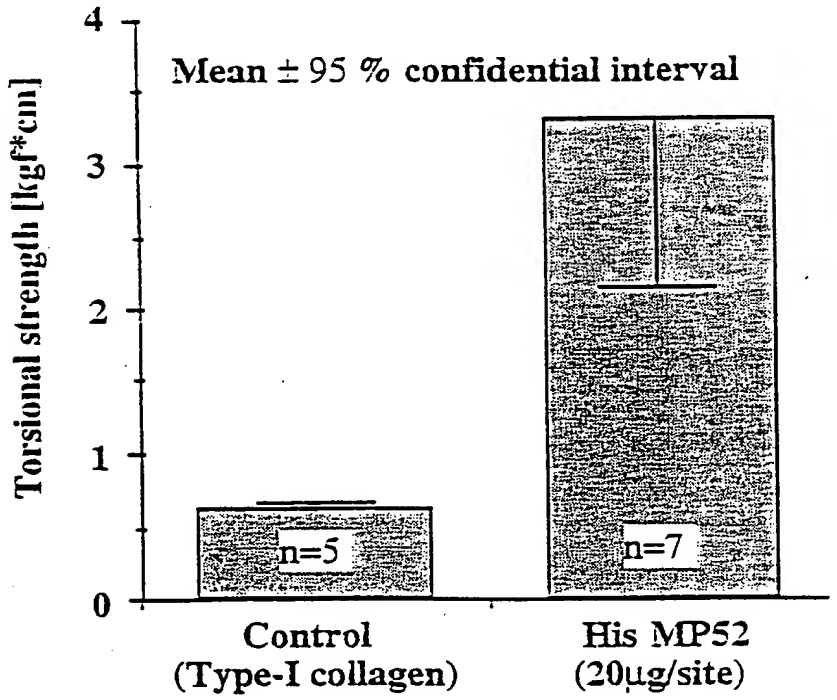

FIGURE 4: Torsional strength of rat femurs with segmental bone defects (5 mm) 12 weeks after implantation of HisMP52 (20 µg/site) with type I

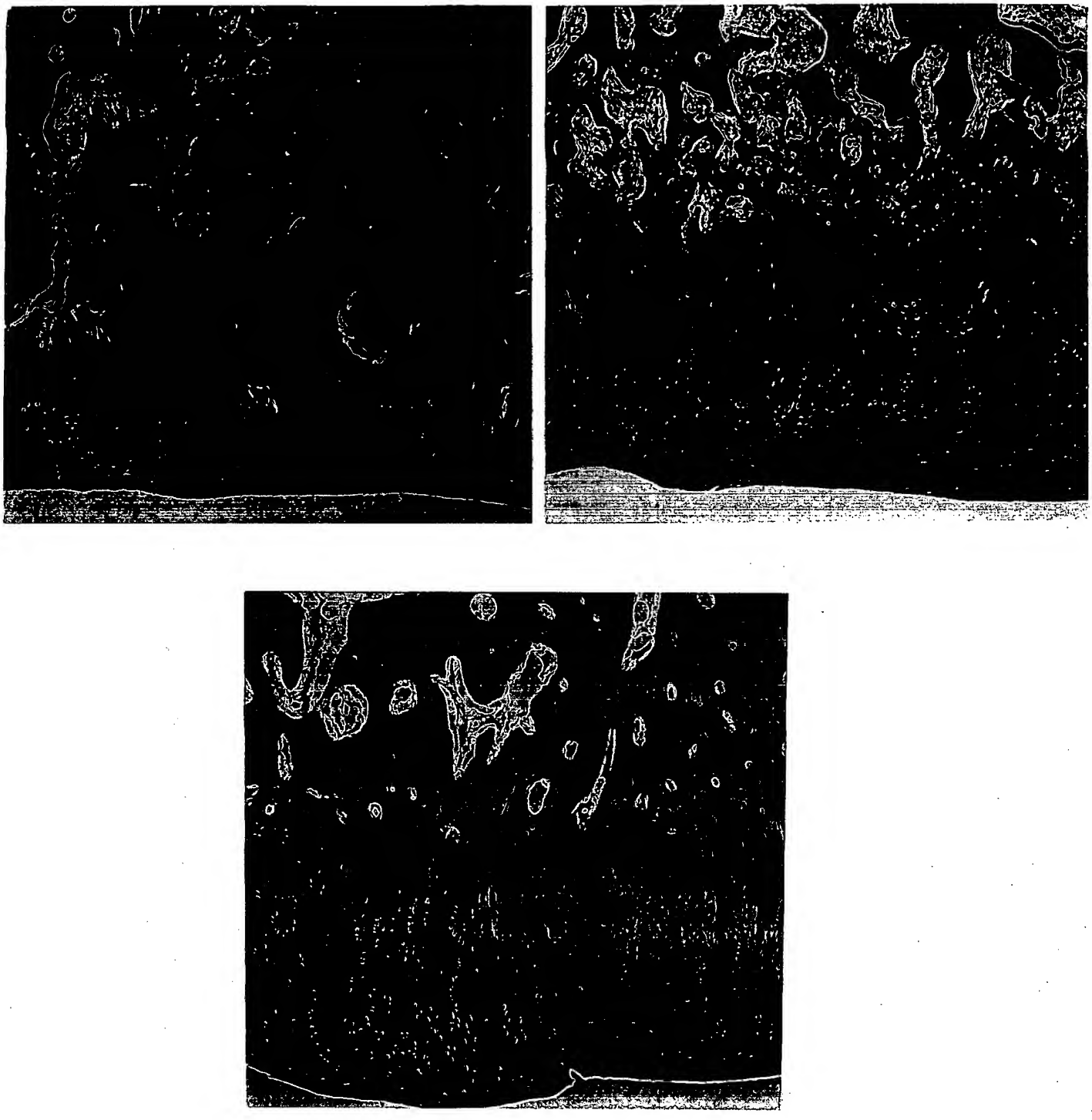

FIGURE 5: Full-thickness defect of articular cartilage in rabbits six weeks after treatment with hyaluronic acid (A) or treatment with a HisMP52/Hyaluronic acid (B) mixture. Intact articular cartilage is shown for comparison (C).

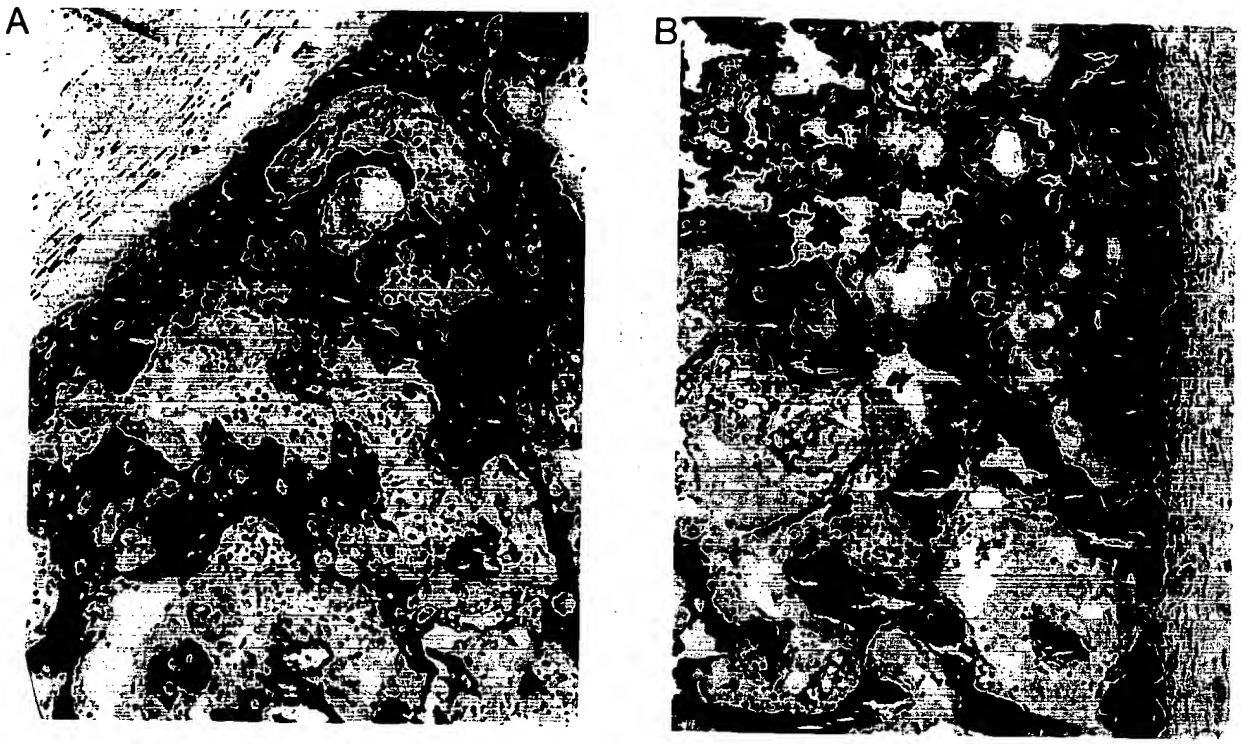

Figure 6: Ectopic bone formation (von Kossa stain) in mice two weeks after implantation of
A: 200 µg rhMP52/ß-TCP (50-150 µm) or
B: 200 µg rhMP52/ß-TCP (150-500 µm).